Exhibit D

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Detection of gene targeting by co-conversion of a single nucleotide change during replacement recombination at the immunoglobulin μ heavy chain locus

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ABSTRACT

A method is described for detecting targeted events at the μ heavy chain gene which relies on co-conversion (or co-exchange) of a point mutation with a selectable marker contained on a replacement vector. The vector, designed for application to IgM producing hybridomas, contains a single nucleotide change within the region of homology with the target gene which encodes a different allotypic determinant of IgM. In a model system where homologous recombination corrected a defective μ gene, the length of homology between this nucleotide change and the position of the double strand break in the vector was found to have a critical influence on the co-conversion frequency. In the vector design ultimately used for targeting in hybridomas, one in 1000 – 2000 stable transformants produced IgM with the allotype encoded by the exogenous DNA, and Southern blot analysis confirmed that these were derived by targeted integration. The sensitivity of the screening procedure using a monoclonal antibody specific to this allotype enabled a targeted clone to be detected in a pool of stable transformants when present at a frequency at least as low as one per cent. Several different modifications of the target locus were obtained as a consequence of alternative crossover positions and, in some cases, vector DNA concatenation.

INTRODUCTION

We describe the construction and testing of a replacement vector designed for targeting the μ locus of IgM secreting cell lines. The design of the targeting vector facilitates rapid identification and isolation of targeted clones based on detection of an allotype change in the expressed IgM.

Site-specific (targeted) integration of exogenous DNA by homologous recombination occurs inefficiently in mammalian cells compared to random integration arising by non-homologous recombination (1–9). Methods for isolating clones harbouring targeted integrations of DNA from the larger fraction of stable transfomants derived by non-homologous integration either involve genetic enrichment for these clones (10,11), or some form of physical screening of the DNA sequences of stable transformants for alterations specific to a targeted event usually combined with a sib-selection strategy (12). An effective alternative to these methods applicable to situations where the target gene is expressed and the gene product is presented on the cell surface or secreted, is to design a vector which will modify the coding region of the target gene such that the cell expresses a novel antigenic component, and can therefore be easily detected with an antibody probe. This approach has been termed 'epitope addition' (13) and involves the use of insertion vectors to introduce a relatively large region of unique sequence encoding the antigenic component and a selectable genetic marker into the target gene. A variation of this strategy is described here using a replacement vector to introduce a point mutation into the μ heavy chain gene of hybridomas resulting in the exchange of a single amino acid residue which is an allotypic determinant of IgM (14). The vector is designed for use with BALB/c derived hybridomas (μ^a allotype) and encodes an amino acid change (Arg to Lys) in the CH1 domain of the constant region that converts the allotype of the IgM to that of the C57/BL6 strain (μ^b allotype). This can be specifically distinguished using the anti-allotype monoclonal antibody MB86 (15) which was produced by generating hybridomas from a BALB/c mouse immunized with C57/BL6 spleen cells.

The design of the vector is explained in Figure 1. The replacement vector DNA contains all the exons of the μ heavy chain constant region and is shown colinear with the functionally rearranged μ gene of a BALB/c derived cell line. A genetic marker, the SV*gpt* gene (16), is located at an internal position just 3′ of exon 4 and does not therefore interrupt the coding sequences required for production of a secreted form of the heavy chain. In the left arm of homology there is a single nucleotide difference with the target sequence, a G to A change in the second position of an AGA codon in exon 1. The nucleotide difference encodes the Arg to Lys amino acid substitution which is the allotypic determinant of the μ alleles of the BALB/c (μ^a) and C57/BL6 (μ^b) strains, respectively. Random integrations of the DNA should not result in any change of the IgM allotype, but a double cross-over at the positions indicated should exchange the exogenous DNA with its cognate sequence and thus cause the cell line to survive the genetic selection and produce an IgM detected with the MB86 monoclonal antibody.

A critical question concerning this strategy is the efficiency with which the point mutation and the selectable marker in the vector will be co-converted (or co-exchanged) into the heavy chain locus. To address this question preliminary experiments with vectors encoding the μ^b allotypic determinant were carried out using a myeloma cell line containing a defective μ^a heavy chain allele, which allowed targeted events to be easily detected by correction of the gene and production of functional IgM (17).

As presented in detail below, in gene correction events the efficiency of conversion of this sequence into the target site was found to depend on the extent of homologous sequence between the AAA codon and the end of the vector arm. On the basis of this result a vector was constructed to enable integrations to be detected at the endogenous μ^a heavy chain locus of a hybridoma using this antibody screening approach. The frequency of the targeting event and the sensitivity of this screening approach for detecting targeted clones in pools of transformants is described. A number of independently derived targeted clones were analysed which revealed that different modifications of the target site can occur. A derivative of the basic vector design was also targeted into the μ locus potentially allowing a positive genetic selection for subsequent rounds of targeting.

METHODS

Construction of plasmids

The vectors used in these experiments were derived from pUC-CμSV*gpt* 2 (17). This contains the heavy chain constant region sequence of the μ^a allele with a SV*gpt* gene located immediately 3' of exon 4. The vector pUC-CμSV*gpt*6 (Figure 2B) is a derivative of this in which a G to A change was made in the AGA codon in exon 1, thus specifying the allotypic determinant of the C57/BL6 strain. This was constructed using a polymerase chain reaction (PCR) site-directed mutagenesis strategy. PCR was carried out on pUC-CμSV*gpt* 2 DNA using an oligonucleotide (5'ACATGCAGATCTTTGTTTTTGCCTCC3') complementary to the sequence around the AGA codon which includes a *Bgl*II site (underlined), but containing the required single base change (bold type); and an oligonucleotide (5'TGATTACGAATTC GAGCTCGCCC3') complementary to the pUC-12 sequence surrounding the *Eco*RI site (underlined) in the plasmid polylinker region. Amplification was carried out using Cetus *Taq* polymerase for only eleven cycles to minimize the possibility of mutations. The resulting 581 base pair PCR product was cleaved with *Eco*RI and *Bgl*II and then ligated into pUC-CμSV*gpt*2 DNA cut at the equivalent *Eco*RI and *Bgl*II sites. The DNA was sequenced in the region of the *Bgl*II site to confirm the mutation had been introduced.

The plasmid pUC-V_{NP}CμSV*gpt*6 (Figure2D) consists of a 3.9 kilobase (kb) *Eco*RI fragment containing a functionally rearranged variable heavy chain coding sequence together with promoter and enhancer sequences, inserted into the *Eco*RI site of pUC-CμSV*gpt*6 adjacent to the 5' side of the constant region. This *Eco*RI fragment was derived from pUC-V_{NP}Cμ*neo* (17) which is the target plasmid shown in Figure 2 and encodes a domain with binding specificity to the hapten 4-hydroxyl-3-nitro-phenacetyl (NIP). It is inserted in the same orientation and thus the sequences in this plasmid encodes a complete μ polypeptide.

The vector pUC-VΔ2CμSV*gpt*6 (Figure 2A) was made by insertion of a 2.3 kb *Eco*RI fragment which contains a 5' deletion of the above variable region sequence, the generation of which has been previously described (17), into the same *Eco*RI site and in the same orientation as above. The plasmid is incapable of producing a functional μ polypeptide.

The vector pUC-SμCμSV*gpt*6 (Figure 3A) was derived by first purifying a 3.5 kb *Eco*RI-*Xba*I fragment from pSV-Vμ1 (18) made by partial *Xba*I and complete *Eco*RI digestion which contains sequence extending from an *Eco*RI site in the heavy chain enhancer to the *Xba*I site close to the 5' side of exon 1 of the μ constant region. This was then inserted into pUC-CμSV*gpt*6 between the *Eco*RI and *Xba*I sites in the polylinker 5' of the coding sequence.

The vector pUC-SμCμSV*gpt*5'Δ*neo*2 (Figure 3A) was derived by inserting a 1.7 kb *Bam*HI cassette containing a 5' deleted *neo* gene into the *Bam*HI site defining the 3' end of the SV*gpt* gene. The 5' deleted *neo* gene was derived from the plasmid pSV2*neo*$^-$ (1) which contains a *Xho*I site in the 5' region of the *neo* coding sequence. This was converted to a *Bam*HI site by insertion of a linker oligonucleotide and the 3' portion of the *neo* gene could then be removed as a *Bam*HI cassette.

Culture, electroporation and selection of cells

The myeloma cell line J558*neo*^{R}1S was used in these studies to determine the efficiency of conversion of the μ^b allotypic determinant. It contains a defective plasmid-derived heavy chain gene pUC-V_{NP}Cμ*neo* and produces no heavy chain. It does however produce a λ1 light chain which contributes to an anti-NIP binding site when the cells are able to express a μ polypeptide encoded by the V_{NP} sequence. Conditions for culture, electroporation and *gpt* selection of these cells, and for the isolation of IgM$^+$ clones by haemolytic plaque assay were described previously (17).

The IgM producing hybridoma cell line used in these targeting experiments was NQ11/14.5 generated from an immune response to the hapten 2-phenyl-5-oxazalone (phOX) (19). This cell line was cloned prior to use in these experiments and a high level producing variant isolated on the basis of haemolytic plaque size. The cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% foetal calf serum, and *gpt* selection was carried out by supplementing this with 250 μg xanthine/ml, 20 μg hypoxanthine/ml and 5 μg mycophenolic acid/ml. For electroporation NQ11 cells were harvested at a density of $2-5\times10^5$ cells/ml, centrifuged at 1000 revs/min and then resuspended in phosphate buffered saline (PBS). They were then centrifuged again as before, and finally resuspended at a density of 10^8 cells/ml in PBS and left on ice. 25 μg of linearized targeting vector was mixed with 0.25 ml of cells (2.5×10^7) and these were electroporated in chilled cuvettes in a Bio-Rad apparatus set at 200V and 250 μF. The cells were left on ice for 30 mins and then added to 25 ml medium/serum. Cells were left for 36–48hr and then plated into *gpt* selective medium/serum at approximately 4×10^5 cells per well of a 24 well plate. This gave between 10–20 *gpt*$^+$ colonies per well. In later experiments cells were divided immediately after each electroporation into four equal portions, and left for 36–48 hours in 25 ml non-selective medium/serum in T25 flasks. This was then replaced by *gpt* selective medium/serum. In either case cells were left in selection for 10 days before sampling of supernatants for radioimmunoassay. Cells from positive wells or flasks were maintained under *gpt* selection and replated in 24 well plates over a range of densities. After screening the supernatants of these wells by radioimmunoassay cells from positive wells were cloned by limiting dilution.

Screening of supernatants by radioimmunoassay

The monoclonal antibodies used in this study were SMI/45 (Rat anti-mouse IgM) (20) and MB86 (15). They were I^{125} labelled by standard procedures (21). Radioimmunoassays were carried out on 96 well polystyrene microtitre plates coated with either 50 μg/ml hapten-coupled BSA or with 50 μg/ml unlabelled MB86 as the capture reagents. The wells were blocked overnight with 0.1% BSA. One hundred microlitres (μl) of supernatant was

added per well and was incubated for 2–3 hours at 37 degrees centigrade. The wells were washed three times with PBS and then 100k c.p.m. of I^{125} labelled antibody added per well. The wells were left for 2–3 hours at 37 degrees centigrade, washed three times with PBS and counted in a gamma counter.

Southern blot analysis

Analysis of genomic DNA by Southern blotting was carried out as previously described (17) except that probes were random primer labelled. Probe A was a 0.9 kb *Hind*III-*Xba*I restriction fragment from pSV-Vμ1 and probe B was a 1.2 kb *Hind*III restriction fragment from pUC-Cμ*neo*.

RESULTS

Frequency of allotype change in gene correction experiments

In order to assess the frequency with which the sequence encoding the allotypic determinant would be transferred into a chromosomal target using the replacement vector strategy outlined in Figure 1, experiments were performed using this vector design in combination with a myeloma cell line containing a defective heavy chain gene. This cell line contains tandem head to tail repeats of a stably integrated plasmid, pUC-V_{NP}Cμ*neo* (17) in which the constant region is disrupted in the second exon by 2 kb of *neo* sequence (Figure 2C). Precise removal of this disruption by replacement recombination with restriction fragments derived from the vectors shown in Figure 2A and B results in production of a complete μ polypeptide with binding specificity to the hapten NIP, encoded by the variable region of the pUC-V_{NP}Cμ*neo* plasmid. These cells also express an endogenous λ1 light chain which associates with this heavy chain and results in secretion of an anti-NIP IgM. Spontaneous and precise removal of the insertion has never been observed.

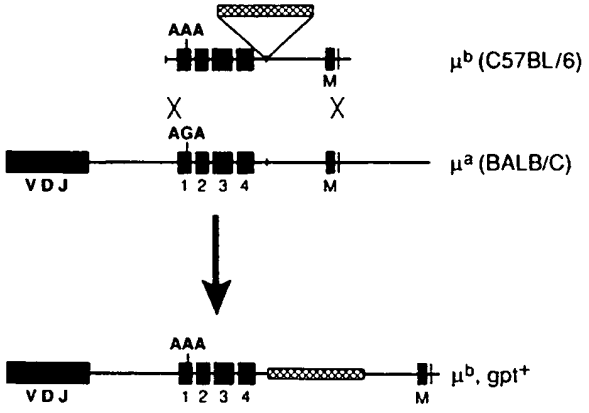

Figure 1. Principle of the method. The replacement vector is designed for targeting the μ constant region of a functionally rearranged heavy chain gene in a BALB/c derived hybridoma (μ^a allele). It contains a SV*gpt* gene located 3' of exon 4 and a single nucleotide difference with the target sequence in the left arm of homology. The nucleotide change in the second position of the AGA codon (G to A; Arg to Lys) specifies a different allotype to that of the BALB/c cell line. If the exogenous μ sequence (μ^b) is exchanged into the chromosomal heavy chain gene by the double cross-over event shown, the cell line will become *gpt*$^+$ and can be distinguished from transformants arising from random integration events by production of a new IgM allotype. The rearranged variable region (VDJ) and constant region exons (1.2.3.4 and M) are denoted by black boxes, and the SV*gpt* gene by a cross-hatched box.

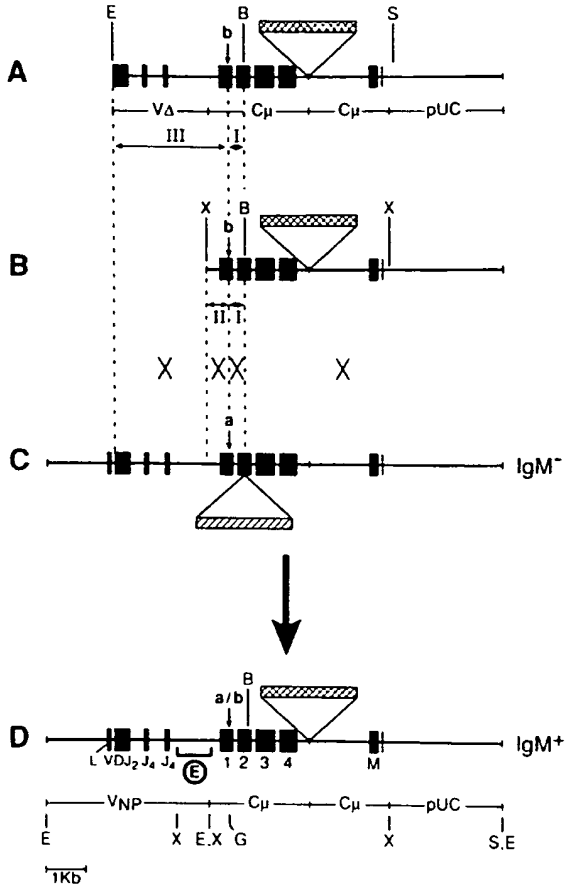

Figure 2. Diagram of the gene correction experiment (A) the targeting plasmid pUC-VΔ2CμSV*gpt*6, (B) the targeting plasmid pUC-CμSV*gpt*6, (C) the plasmid-derived defective heavy chain target gene pUC-V_{NP}Cμ*neo*, and (D) the plasmid-derived corrected heavy chain gene pUC-V_{NP}CμSV*gpt*6, are shown aligned by homology. The exons of the leader (L) and rearranged variable region (VDJ2) sequences, and the exons of the constant region (1, 2, 3, 4,) including the membrane exons (M) are shown by black boxes. The *gpt* gene is shown by a crosshatched box, and the *neo* gene is shown by a shaded box. E defines the position of the heavy chain enhancer. In the case of (A) and (B) the sequence corresponding to the position of the *neo* gene in the target is indicated by the position of the *Bam*HI site. The only other restriction sites shown in (A) and (B) are the sites used to cleave the targeting fragments from the plasmid backbones. b and a define the positions of the sequences encoding the allotypic determinants of the μ^a and μ^b alleles respectively. I, II, III, define the lengths of homology with the target between the *Bam*HI site and the position of the allotypic determinant, and between the allotypic determinant and the left end of the targeting vectors. The sizes of these regions are 0.33 kb, 0.54 kb and 2.8 kb respectively. In a replacement event a cross-over must occur in one of these regions together with a cross-over in a region to the right of the *neo* gene in order to correct the target and produce an IgM$^+$cell. The cross-over to the right of the *neo* gene must occur to the right of the *gpt* gene to give IgM$^+$ cells in experiments involving *gpt* selection, but can occur on either side to produce IgM$^+$ cells isolated directly by haemolytic assay. The result of the targeted event is shown in (D) in which the sequence of the allotypic determinant (shown as a/b in the diagram) depends in principle on the position of the left hand cross-over. The location of other restriction sites in the variable region segment (V_{NP}), constant region segment (Cμ), and pUC sequences, pertinent to understanding the construction of all these plasmids are shown only in (D) to maintain clarity (it should be noted however that the *Eco*RI site delineating the V_{NP} and Cμ segments was removed from the construct shown in (A) and an *Eco*RI site inserted in this construct at the 3' end of the deletion in the V_{NP} sequence). Restriction enzymes are abbreviated as follows: B, *Bam*HI; E, *Eco*RI; G, *Bgl*II; S, *Sal*I; X, *Xba*I.

Homologous recombination events can be detected by plating the transfected cells directly after recovery from the electroporation treatment, or after growth in selective medium, into soft agar with hapten coupled Sheep Red Blood Cells (SRBCs) (17). Addition of guinea pig complement 48 hours after plating gives rise to clear areas due to haemolytic lysis in the vicinity of any targeted cells. These can therefore be identified, cloned and subsequently analysed.

The targeting vectors pUC-CμSV*gpt*6 and pUC-VΔ2CμSV*gpt*6 are shown in Figure 2 aligned with the pUC-$V_{NP}C\mu neo$ plasmid target. These are identical to targeting vectors previously described (17) which can recombine with this defective constant region and exchange their normal μ sequence with the disrupted copy by a double crossover event, but are modified by the single nucleotide substitution G to A to encode the μ^b allotypic determinant. The target DNA encodes the μ^a allotypic determinant. The position of the contiguous μ sequence in the vectors which must be exchanged to restore the coding sequence is marked in Figure 2 by a *Bam*HI site, and the distance (I) between this and the nucleotide sustitution is 333 bases. By isolating IgM$^+$ cells from transfections with these vectors and screening for the allotype of the IgM it was therefore possible to measure the frequency with which these two sequences co-segregated in the gene correction event.

In this experiment restriction fragments were prepared from the above plasmids and were then gel purified from the pUC backbone. The restriction fragments used were the 6.5kb *Xba*I fragment from pUC-CμSV*gpt*6 and the 10.5 kb *Eco*RI-*Sal*I fragment from pUC-VΔ2CμSV*gpt*6 (see Figure 2). The distance (II) between the G to A substitution and the left end of the *Xba*I CμSV*gpt*6 fragment is 535 bases, and the distance (III) between the substitution and the left end of the *Eco*RI-*Sal*I fragment is approximately 2.8 kb. J558*neo*^{R}1S cells were electroporated with these restriction fragments and IgM$^+$ clones were identified by complement mediated haemolysis either by first selecting in *gpt* selective medium for ten days and then plating into soft agar at low cell density ($10^4 - 10^3$ cells per 3.5 cm diameter well), or by direct plating into soft agar two days after electroporation at high cell density (5×10^5 cells per 3.5 cm diameter well). In the case of transfections with the *Xba*I CμSV*gpt*6 fragment both procedures were used, and a total of 17 clones were isolated from four independent transfections, three of which involved *gpt* selection. A consistent observation with both protocols was the lower frequency with which IgM$^+$ clones were identified compared to transfections done in parallel with the CμSV*gpt*2 *Xba*I fragment. For example after *gpt* selection IgM$^+$ clones were identified in soft agar at an average fequency of 0.77% of *gpt*$^+$ transformants derived using the CμSV*gpt*6 fragment, compared to 1.37% with the CμSV*gpt*2 fragment. In Table 1 a comparison is shown of targeting efficiencies obtained with the *Xba*I CμSV*gpt*2 and CμSV*gpt*6 restriction fragments in three independent experiments using the direct plating assay. With the *Sal*I-*Eco*RI VΔ2CμSV*gpt*6 fragment a total of 14 IgM$^+$ clones were isolated from two independent transfections using the direct plating strategy, which is more convenient in the case of this fragment since the absolute targeting efficiency is approximately ten fold higher and many clones can be picked from a few wells (average of 7 per well; targeting efficiency = 1.4×10^{-5}). The targeting efficiency in this case was the same as for the equivalent fragment without the G to A mutation. In most cases IgM$^+$ isolates obtained from these transfections were replated in soft agar to ensure that pure clones were obtained since they can have a growth disadvantage compared to IgM$^-$ J558*neo* ^{R}1S cells. Clones were expanded into culture and supernatants taken when the medium had turned acidic. J558*neo*^{R}1S cells were also transfected with the plasmid pUC-$V_{NP}C\mu$SV*gpt*6 which contains a complete functional heavy chain variable region linked to the constant region sequence encoding the μ^b allotypic determinant. This plasmid should produce a functional μ polypeptide irrespective of its mode of integration and therefore provided supernatant for use as a positive control.

Table 1.

	CμSV*gpt*2	CμSV*gpt*6
1	4.0×10^{-6}	0.9×10^{-6}
2	1.3×10^{-6}	0.3×10^{-6}
3	2.6×10^{-6}	1.6×10^{-6}

Comparison of targeting efficiencies obtained with the 6.5 kb *Xba* I fragments of pUC-CμSV*gpt*2 and pUC-CμSV*gpt*6 transfected into the J558*neo*^{R}1S cell line. Three experiments were done in which the two fragments were transfected in parallel. Cells were plated directly into soft agar (48 hours after electroporation) with NIP-coupled SRBCs and complement added 48 hours later. The targeting efficiency is calculated as the ratio of the total number of plaques identified divided by the total number of viable cells plated. In each experiment a separate aliquot of the transfected cells were plated into agar with *gpt* selective medium and colonies counted after ten days; the efficiencies of *gpt* transformation calculated on this basis were approximately the same for the two fragments in a single experiment and their relative values did not vary between experiments, thus excluding the possibility that the different values for the targeting efficiencies resulted from inaccuracies in the amounts of DNA used.

Supernatants collected from IgM$^+$ clones were adsorbed onto wells coated with NIP-BSA and sets of wells with each supernatant were probed with I^{125} labelled MB86 to detect IgM of the μ^b allotype, and with I^{125} labelled SM1/45 to provide a relative measurement of total IgM. In Table 2 representative results obtained with these clones, and with the positive and negative controls are presented. The data allowed clones with IgM of the μ^b allotype to be unequivocally distinguished. All of the IgM$^+$ clones derived using the *Sal*I-*Eco*RI VΔ2CμSV*gpt*6 fragment were of the μ^b allotype, compared to only 4 out of the 13 derived using the *Xba*I CμSV*gpt*6 fragment. Furthermore it was clear that the assay was capable of distinguishing supernatant from the μ^b clones even when diluted with supernatant from cells making the IgM of the μ^a allotype, and would therefore provide a sensitive screen with at least small pools of cells.

Thus in conclusion, although the nucleotide substitution is in close proximity to the *Bam*HI site in the targeting fragments the distance from its position to the end of the vector arm appears to influence its co-segregation frquency during gene correction. In the case of those clones isolated with the direct plating strategy the frequency of co-segregation of the *gpt* marker was also assessed by testing for growth in medium with mycophenolic acid, and consistent with our previous observations (17) one third of the clones were *gpt*$^-$, indicating that in these the cross-over point in the right arm of homology was to the left of the *gpt* gene (see Figure 2).

Transfection and screening of hybridoma cells

The above results suggested that successful targeting of an endogenous μ gene using this approach would require a modification of the pUC-CμSV*gpt*6 vector to extend the homology on the left arm. In a functionally rearranged endogenous μ heavy chain locus the constant and variable region

Table 2.

	c.p.m. bound on NIP-BSA coated wells	
	$+I^{125}$-MB86	$+I^{125}$-SM1/45
1	19896	43879
2	1898	36214
3	2020	1254
4	2466	38133
5	1893	36765
6	2308	32708
7	2333	36637
8	2048	35116
9	22990	38459
10	13922	44001
11	8623	44164
12	3641	1187
13	28355	26013
14	11505	20269
15	18185	24848
16	513	18867
17	4535	9241
18	213	164

1 and **13**-supernatants used as a positive control from IgM^+ cells derived by transfection with pUC-$V_{NP}C\mu SVgpt6$; *2*-supernatant used as a negative control from J558*neo*^{R}1S IgM^+3B2 cells (derived from targeting J558*neo*^{R}1S cells with pUC-CμSV*gpt2*, which therefore produce IgM of the μ^a allotype) (17); *3*-supernatant used as a IgM^- control from untransfected J558*neo*^{R}1S; **2** to **8**, and **16**-IgM^+ clones derived from targeting with pUC-CμSV*gpt6* which are negative for conversion of the allotype; *9*- IgM^+ clone derived from targeting with pUC-CμSV*gpt6* which is positive for conversion to the μ^b allotype; **10** and **11**-1/10 and 1/25 dilutions of supernatant from **9** made with supernatant from the J558*neo*^{R}1S IgM^+3B2 culture; **14** and **15**-IgM^+ clones derived from targeting with pUC-VΔ2CμSV*gpt6*; **12** and **18**-background controls using medium/5% foetal calf serum. Results from wells with positive values in the assay are underlined. The results in **1** to **11** and **12** to **18** were obtained using different batches of the Iodinated antibodies which have slightly different relative specific activities, and also give different background levels.

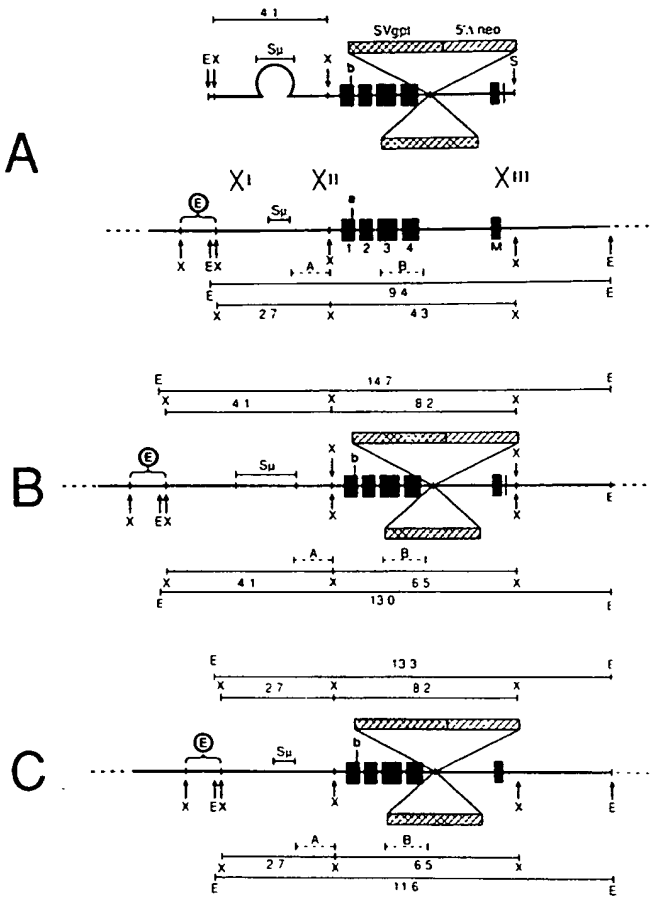

Figure 3. Structure of the vectors for targeting the μ locus and the predicted rearrangements from replacement recombination events. **(A)** The structure of the pUC-SμCμSV*gpt6* and pUC-SμCμSV*gpt5'*Δ*neo2* vectors after removal from the plasmid backbone by *Eco*RI and *Sal*I cleavage, are aligned with and above the chromosomal μ heavy chain sequence extending from the position of the enhancer to the *Eco*RI site 3' of the membrane exons. The vectors are identical with a 2.2 kb SV*gpt* gene inserted 3' of the exon 4 sequences, but pUC-SμCμSV*gpt5'*Δ*neo2* also has a defective (5' deleted) 1.7 kb *neo* gene inserted in tandem with the SV*gpt* gene. For reasons of space the SV*gpt* and SV*gpt5'*Δ*neo* cassettes are shown respectively, above and below the same vector Cμ sequence with their position of insertion indicated. The larger region of Sμ sequence in the vector relative to the target (see text) is shown looped out to preserve the alignment of the sequences. The positions of cross-overs which can explain the rearangements in the targeted lines are shown and distinguished I, II, and III. **(B)** The predicted structure of the targeted locus resulting from replacement recombination with the vectors in **(A)** in which cross-overs have occurred at positions I and III. The SV*gpt* and SV*gpt5'*Δ*neo* cassettes are shown respectively, above and below the chromosomal Cμ sequences. In this recombination event the size of the Sμ sequence is expanded. **(C)** Predicted structure of the targeted locus after replacement recombination with both vectors in which cross-overs have occurred at positions II and III. The SV*gpt* and SV*gpt5'*Δ*neo* cassettes are shown as above. In this recombination event the size of the Sμ region remains the same. Exon sequences are indicated by black boxes, the SV*gpt* gene by a cross-hatched box, the defective 5'Δ*neo* gene by a stripped box. The positions of the sequences encoding the μ^a and μ^b allotypic markers are indicated by **a** and **b** respectively. The regions complementary to the probes **A** and **B** used in the Southern blotting are shown by a dotted line, the region of the Sμ sequence by a solid line, and the position of the heavy chain enhancer by **E**. Restriction enzyme abbreviations are as in the legend to Figure 2 and the fragments generated by these enzymes which are detected with the probes **A** and **B** shown by solid lines with the size in kb.

sequences are separated by a noncoding region which contains the heavy chain enhancer and a region of short highly repeated DNA sequences, the switch (Sμ) region (22) involved in recombination during heavy chain class switching. The left arm of homology of the pUC-CμSV*gpt6* targeting vector was therefore extended beyond the *Xba*I site, by the addition of a 4.4 kb *Eco*RI-*Xba*I restriction fragment obtained from the vector pSV-Vμ1 (18) containing sequences equivalent to part of this region. The structure of the resulting targeting vector pUC-SμCμSV*gpt6* is shown in Figure 3A (without the plasmid backbone which is removed by *Eco*RI and *Sal*I cleavage prior to transfection), aligned with the target locus. The size of the targeting vector is 10.6 kb, with a left arm of homology of 6.8 kb and a right arm of homology of 1.9 kb. The region of additional sequence extends right to left across the repeated DNA sequences of the Sμ region to a position in the Ig heavy chain enhancer. The boundaries of the Sμ region indicated in Figure 3 relative to the enhancer and constant region are based on the sequence and mapping data of Nikaido *et al.* (23). Because the sequence of the Sμ region is comprised of multiple repeats of two simple sequences internal deletions occur probably due to homologous recombination during propagation in *E. coli*, and in this vector an *Xba*I fragment, for example, which encompasses this region is 4.1 kb (see Figure 3A) compared to approximately 6.0 kb in mouse germ line DNA. However in the hybridoma NQ11/14.5 which was chosen for use in this experiment (see below) this

restriction fragment is contracted from the germ line size by an even greater extent than in the cloned DNA, to 2.7 kb. In the alignment of homologous vector and target sequences shown in Figure 3 the left arm of the targeting vector is looped out at the position of the Sμ region on the assumption that the size difference is the consequence of similar recombination events in this region in the cell line which have occured during protracted culture.

NQ11/14.5 is an IgM producing hybridoma derived from a BALB/c mouse immunized with the hapten 2-phenyl-5-oxazolone (phOx) (19). A clone of this cell line was used which gives a large plaque size in *in-situ* haemolysis. For a single transfection 2.5×10^7 NQ11/14.5 cells were electroporated with 25 μg of the above linearized construct, and were left in non-selective medium for 48 hours. After this time the viable cell count was usually $2-3\times10^7$. In initial experiments cells were plated into *gpt* selective conditions in 24 well plates at a density of 4×10^5 cells per well (usually 48−60 wells per transfection). 10−20 gpt^+ colonies were obtained per well after 10 days which is a transformation efficiency of 2.5×10^{-5}–5×10^{-5}. In the first round of experiments supernatants were analysed by radioimmunoassay from independent platings of six electroporation experiments. In the case of two of these transfections, a small fraction of the wells in each gave signals which were significantly above the background level. Examples of positive and negative wells in this initial screening strategy are shown in Table 3. The cells from some of these positive wells (e.g. 6-15, 2-1 and 2-4) were then replated at lower density. Supernatants from these wells were reanalysed and some of these gave high signal values comparable to or higher than the initial signal. Cells from these wells were cloned, expanded and genomic DNA prepared. Southern blot analysis of DNAs from the clones obtained in these two transfections confirmed that the endogenous locus had been modified by a replacement recombination event (see below). In an equivalent number of transfections using the *Xba*I CμSV*gpt*6 fragment no positive wells were obtained by the radioimmunoassay screening which was not unexpected given the result described in the previous section.

The sensitivity of the radioimmunoassay screen was tested to determine the detection limit when a targeted clone was present in different pool sizes of stable transformants. The mixing experiment shown in Figure 4 was therefore carried out in which one of the targeted clones isolated in the above experiment was co-plated with untransfected NQ11/14.5 cells over a range of ratios of the two cell types, but at the same final density of approximately 5×10^5 cells per well. After allowing the cells to grow for two days supernatants were assayed as described above. The results show that even when the targeted clone was mixed with NQ11/14.5 cells at a ratio of 1 to 100 a significant signal above background was seen with both radioimmunoassay protocols, although adsorbtion to MB86 coated wells and probing with SM1/45 gave a stronger signal presumably because there is no competition for binding in this version of the assay. A signal above background was seen at a one to 1000 ratio, but this was not considered reliable for routine screening. The sensitivity of the assay therefore enabled individual transfections to be rapidly screened for the generation of a targeted clone by dividing cells

Table 3.

	c.p.m. bound on phOX-BSA coated wells		c.p.m. bound on MB86 coated wells
	$+I^{125}$-MB86	$+I^{125}$-SM1/45	$+I^{125}$-SM1/45
NQ11	330	28393	301
6-2	443	32127	496
6-10	367	34380	685
6-15	29532	30008	25894
6-18	396	31607	489
2-1	5486	28737	11488
PBS	573	779	402
2-D/1	77	ND	15917
2-1	14587	ND	15817
2-2	109	ND	530
2-3	84	ND	599
2-4	12374	ND	15636
2-5	52	ND	1370
5%FCS	22	ND	24

Supernatants incubated in wells coated with phOX-BSA as the capture reagent were probed with I^{125}-labelled MB86 to identify IgM of the μ^b allotype, and in some cases with I^{125}-labelled SM1/45 to obtain a relative measure of total IgM (this was a useful control to ensure no positive wells were missed in the assays on account of low cell density). Supernatants incubated in wells coated with MB86 as the capture reagent were probed only with I^{125} labelled SM1/45. The supernatants 6-2, 6-10, 6-15, 6-18 and 2-1, 2-2, 2-3, 2-4, 2-5 were taken from wells containing gpt^+ colonies of NQ11/14.5 cells from two independent transfections with the *Eco*RI-*Sal*I fragment of pUC-SμCμSV*gpt*6. Supernatant from untransfected NQ11/14.5 cells was used as a negative control, and supernatant from 2-D/1 (a J558neo^R1S targeted clone producing IgM of the μ^b allotype) used as a positive control with wells coated with MB86 as the capture reagent.

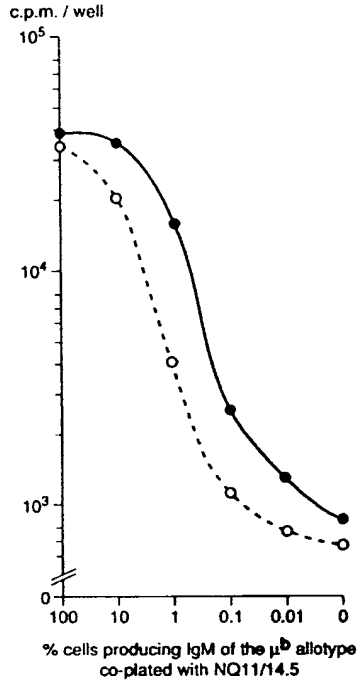

Figure 4. Detection limit of the screening method. Different numbers of cells from one of the positive clones isolated in the initial round of transfections were mixed with NQ11/14.5 cells so they were present at 10, 1, 0.1 and 0.01% of the total and these were then plated in 24 well plates at a final cell density of 5×10^5 per well. Wells were also plated with positive cells alone and with NQ11/14.5 cells alone at the same final cell density. After two days supernatants were incubated in phOX-BSA coated and MB86 coated polystyrene microtitre plate wells, and probed with I^{125} labelled MB86 and I^{125} labelled SM1/45 respectively, as described. Filled circles and solid line- c.p.m. bound using MB86 coated wells; open circles and dotted line- c.p.m. bound using phOX coated wells.

immediately after electroporation into four equal portions in flasks. After allowing cell recovery and growth for 36–48 hrs *gpt* selection was applied. On the basis of the transformation efficiency given above and the viability of the cells after electroporation which was about 40% it was calculated that each flask contained approximately 60–120 independently derived transformants. Thus if a targeted clone was present it would be well within the limits of detection of the assay, with some allowance for any potential growth disadvantage. After maintaining selection for 10 days samples of the supernatant from each flask were assayed and on average one flask gave a positive signal for every four transfections. This gives a frequency of targeting in the range of 10^{-3} to 2×10^{-3} of the stable transformants, assuming that all cells positive for MB86 binding are bona fide targeted events. Cells from positive flasks were then replated into selective medium in 24 well plates over a range of densities. Cells from positive wells from this plating at the lowest density were then replated in selective medium at cloning densities. The remainder of the targeted clones described in this paper were isolated by this protocol including clones from transfection with the pUC-SμCμSV*gpt5'*Δ*neo2* vector which were recovered at a similar frequency.

Analysis of the recombination events by Southern blotting

The structure of the μ locus was analysed by Southern blotting in four cell lines (2-4, 6-15, B2-37, and B4-g) obtained by transfection with the pUC-SμCμSV*gpt*6vector and two lines (F4–2, J3-d) obtained by transfection with the pUC-SμCμSV*gpt5'*Δ*neo2* vector, which were all positives isolated by the radioimmunoassay screen. The sizes of *Eco*RI and *Xba*I restriction fragments in the targeting vector and the unmodified locus, and the predicted sizes of fragments resulting from targeted events using these two vectors are shown in Figure 3. Figure 5A shows *Xba*I digests of these DNAs hybridized with probe A (complementary to sequences in exons 3 and 4) which detects a 4.3 kb band in NQ11/14.5 DNA (lane i). There is only one copy of the heavy chain locus in the hybridoma and thus a targeted event should result in the disappearance of this 4.3 kb band and the appearance of a band representing a fragment of 6.5 kb or 8.2 kb due to insertion of the SV*gpt* or SV*gpt5'*Δ*neo* cassettes respectively (i.e. the size of the XbaI fragments from digests of the plasmid standards in lanes a and h in Figure 4A). The 4.2 kb band is absent in all of the clones except F4-2. In clones 2-4 and B4-g there is a new band with a size of 6.5 kb (lanes b and e) and in clone J3-d there is a new band with a size of 8.2 kb (lane g); the intensities of these bands are equivalent to single copy level and no other bands are present. Thus these are examples of the predicted replacement event obtained with both types of vector. In clone 6-15 a new band of 6.5kb is seen but another band is also present with a size of approximately 9.4 kb, both of which are at single copy intensities (lane c). In clone B2-37 a band of 6.5 kb is also present but it is at approximately three times single copy intensity, and there is in addition a band present of slightly increased size which is at single copy intensity (lane d). These clones are therefore examples where integration of additional copies of vector DNA has occurred along with the anticipated replacement event, although it is not possible to distinguish from this restriction digest whether the additional copies have co-integrated with the homologously recombined DNA, or have integrated at other chromosomal sites.

Figure 4B shows the result after stripping this filter and rehybridizing with probe **B** which detects the *Xba*I restriction

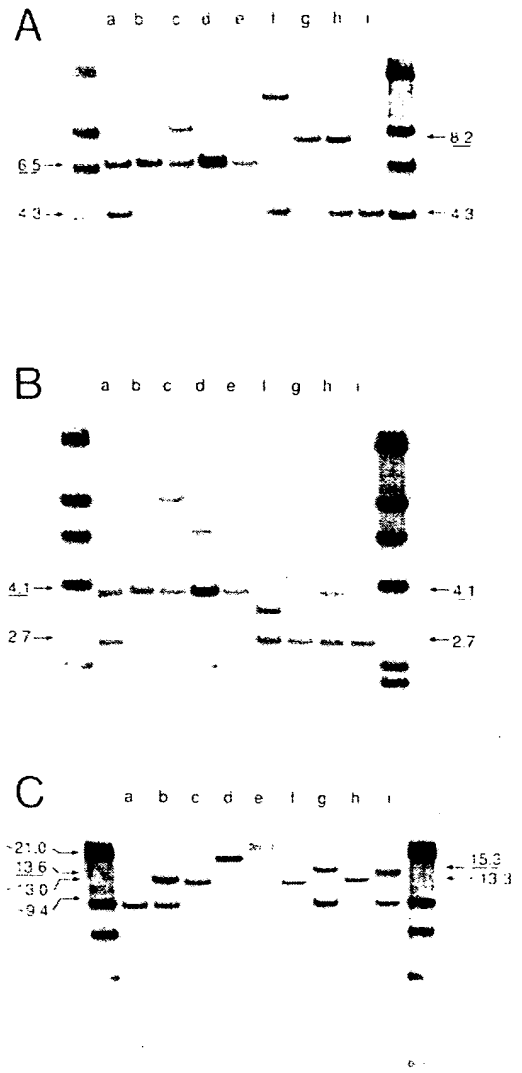

Figure 5. Southern blot analysis of targeted clones. (**A**) *Xba*I digests of genomic DNA from: lane (a) NQ11/14.5 with 10 pg pUC-SμCμSV*gpt*2 plasmid, lane (b) clone 2-4, lane (c) clone 6-15, lane (d) clone B2-37, lane (e) clone B4-g, lane (f) clone F4-2, lane (g) clone J3-d, lane (h) NQ11/14.5 with 10 pg pUC-SμCμSV*gpt5'*Δ*neo2* plasmid, lane (i) NQ11/14.5. The DNAs were run on a 0.7% gel. The filter was hybridized with probe **B** (see Figure 3). The positions of the band representing the endogenous *Xba*I fragment (4.3 kb), and the bands of 6.5 kb and 8.2 kb derived from the plasmid standards are shown. (**B**) The filter from above was stripped and rehybridized with probe A (see Figure 3). The positions of the band representing the endogenous *Xba*I fragment (2.7 kb) and the 4.1 kb band derived from both the plasmid standards are shown. (**C**) *Eco*RI digests of genomic DNAs from: lane (a) NQ11/14.5, lane (b) NQ11/14.5 with 10 pg pUC-SμCμSV*gpt*2 plasmid, lane (c) clone 2-4, lane (d) clone 6-15, lane (e) clone B2-37, lane (f) clone B4-g, lane (g) clone F4-2, lane (h) clone J3-d, lane (i) NQ11/14.5 with 10 pg pUC-SμCμSV*gpt5'*Δ*neo2* plasmid. The DNAs were run on a 0.7% gel. The positions of the bands corresponding to the 13.6 kb and 15.3 kb *Eco*RI unit sizes of the plasmid standards, the endogenous *Eco*RI fragment of approximately 9.4 kb (in lanes a, b, g and i), and the novel fragments of approximately 13.0, 13.3, and 21.0 kb (in lanes b, d and g respectively) are shown. *Hind*III digested λ DNA was run on the outside lanes of each gel, and the hybridizations included a λ DNA probe. The sizes of these fragments are 23.1, 9.4, 6.6, 4.4, 2.3 and 2.0 kb. The sizes of fragments derived from the plasmid standards are underlined.

fragment extending from a position just 5' of the Cμ exon 1 sequence to a position close to the left end of the targeting fragment. The size of this fragment is 2.7 kb in the NQ11/14.5 DNA (lane i) and 4.1 kb in the targeting vectors (lanes a and h). As discussed this restriction fragment encompasses the Sμ region and it is likely that the variance in the size is a consequence of deletions of the Sμ repeats. Depending on the position of the left hand cross-over junction the additional sequence in the targeting vector may or may not be incorporated at the target site. In the case of clones 2-4, 6-15, B2-37, B4-g the 2.7 kb *Xba*I fragment is absent but a new band is present with a size identical to that in the targeting vector (lanes b, c, d and e respectively). Thus in the case of these clones the data is consistent with the left hand cross-over position occurring within region I in Figure 3A to give the arrangement of sequences shown in Figure 3B. In the case of clones 6-15 and B2-37 probe A also detects bands of completely unexpected sizes, and in the case of clone B2-37 the 4.1 kb fragment is present at approximately three times single copy intensity, therefore paralleling the situation observed with probe B. In clone J3-d the 2.7 kb fragment is still present (lane g), and therefore the left hand cross-over probably occurred in region II to give the arrangement of sequences shown in Figure 3C.

*Eco*RI digests of these DNAs hybridized with probe A (Figure 5C) are informative since there are no internal *Eco*RI sites in the targeting fragment. Thus any replacement event should result in an increase in size of the *Eco*RI fragment of approximately 9.4 kb detected with this probe in NQ11/14.5 DNA (lane a). In the case of clones 2-4 and B4-g, the band representing the 9.4 kb restriction fragment is replaced by one representing a size of approximately 13.0 kb (lanes c and f). This is entirely consistent with the previous data and the generation of these clones by a replacement event using the pUC-SμCμSV*gpt*6 vector with cross-overs at positions I and III (see Figure 3B). In clone J3-d obtained from transfection with the pUC-SμCμSV*gpt*5'Δ*neo*2 vector the band representing the 9.4 kb restriction fragment is also replaced, but by one of slightly larger size representing a fragment of approximately 13.2−13.5 kb (lane h). This is again entirely consistent with the previous data and with a cross-over in the left arm of the targeting vector in region II which does not therefore increase the length of the switch region (see Figure 3C). In clones 6-15 and B2-37 there is a single band but of substantially larger size than in the other clones (lanes d and e). Taken together with the result of the *Xba*I digests which indicated the presence of more than one copy of the vector sequences in these clones and the fact that no other bands are seen in the *Eco*RI digests, this indicates that integration of several copies of the targeting fragment has taken place at the homologous site. This could occur for example by end to end joining of copies of the targeting fragment to form a concatenate, with the ends of the concatenate then participating in a replacement type event integrating the entire structure. The size of the fragment in clone 6-15 is 20−22 kb and the presence of an extra band of equal intensity in the *Xba* I digests hybridized with both probes A and B indicates that in addition to the predicted replacement event there may have been insertion of one other additional copy. If for example two copies of the targeting fragment joined in a head to tail configuration and the ends of this cocatenate were involved in the double cross-overs this would result in a size increase which was the sum of the predicted size increase of 3.6 kb (see Figure 3C) and the size of the targeting vector, 10.9 kb, which would result in a net increase of 14.5 kb to give a new fragment of 24.1 kb. This is slightly larger than that observed but end to end joining may involve some deletion and alterations to the structure of the targeting vector and therefore give rise to unpredictable size changes and the loss of restriction sites (which would also explain the novel sizes of the additional *Xba*I fragments). In the case of clone B2-37 the intensity of the bands in the *Xba*I digests suggests the presence of two to three additional copies and the size of the EcoRI fragment is accordingly substantially larger but beyond the effective resolution of the gel.

The *Xba*I digests were not consistent with clone F4-2 having being derived from a targeted event, and the *Eco*RI digest is in support of this since the endogenous band remains in this clone with a new band representing a fragment size of approximately 16 kb (lane h).

CONCLUSIONS

This work demonstrates the feasibility of detecting targeted integrations at the endogenous μ locus by screening for a point mutation in the coding sequence which can co-segregate during the homologous recombination with the genetic marker used to select the transformants. The targeting vector was designed on the basis of information obtained from studying recombination at a plasmid-derived μ target sequence in which the recombination event replaced a disrupted μ sequence with normal sequence. Although in these experiments the allotype marker is closely linked to the contiguous μ sequence (marked by the *Bam*HI site in Figure 2), which must be exchanged to produce a functional heavy chain the frequency of co-segregation was less than 25% for the targeting fragment (CμSV*gpt*6) with the short left arm, but increased to 100% for the targeting fragment (VΔ2CμSVgpt6) with the long left arm. Consistent with these observations are recent studies (24) using gapped insertion vectors designed for targeting the *hprt* locus which have shown that when vector-carried heterologies are not more than 0.8 kb from a gap or break they are usually not retained, and that the frequency is improved the further these are located from the double strand break point. There are a number of potential explanations for the low frequency of co-segregation of the markers in the gene correction experiment using the short arm vector, none of which is mutually exclusive. The simplest, is that the ends of the targeting fragment become exonucleolytically degraded such that, either the sequence encoding the allotypic determinant is actually removed, or insufficient homology remains on its left side to allow formation and/or resolution of a cross-over junction which will incorporate it into the target. However it was shown in our previous work (17) that 220 bases of sequence homology between the *Bam* HI site and the left end of the targeting vector was sufficient for the targeting reaction to proceed and it seems unlikely that this fragment would work at all in a targeting reaction if such extensive and efficient degradation of duplex ends is occurring. The second explanation is that the intervening region between the allotype marker and the *neo* disruption is a preferred site for a cross-over junction on this arm of the vector and thus the sequence for the allotype marker is excluded in the exchange of vector sequences with the target. If the region of homology is increased this may provide alternative and competing sites for cross-overs therefore resulting in incorporation of the allotype marker. A third explanation is that cross-over events may be initiated at the ends of the vector arm but extensive branch migration occurs prior to resolution and that correction of the

resulting heteroduplex DNA at the allotype marker is predominantly in favour of restoration of the μ^a sequence. If the region of homology is increased it could be argued that there is substantially less probability that branch migration will proceed this increased distance from the end of the fragment to the allotype marker position. Bias in terms of repair of mismatches in heteroduplex DNA in a targeting event has been reported in experiments to introduce point mutations into the RNA polymerase II gene in embryonal stem cells (25). In these experiments a marker on the targeting vector defined by a single nucleotide change from the chromosomal sequence (C to T on the coding strand) which was closely linked (30 bp) to an α-amanitin resistance mutation co-segregated at only 30–40% efficiency. Heteroduplex formation at the site of the marker results in a GT mismatch if the sense strand of the vector invades the target, or a CA mismatch if the anti-sense strand invades. Approximately 95% of GT mismatches are corrected in mammmalian cells and >90% of these are corrected to GC, whereas there is no directional bias for AC mismatches (26, 27, 28). It was proposed that the low co-segregation frequency was a reflection of GT to GC correction which restores the chromosomal sequence. In the experiments described here the nucleotide change between target and vector sequence is G to A in the coding or sense strand and thus GT mismatches will result when the anti-sense strand of the targeting vector is the invading strand. To explain the low frequency of co-segregation in the gene correction experiment on this basis we would have to argue that it is the anti-sense strand on the left arm of the targeting vector which is predominantly the invading strand (i.e. the strand ending 3′ at the double strand break), therefore resulting in a high frequency of restoration as a consequence of GT to GC correction bias.

The frequency with which the CμSV*gpt*6 fragment targeted the defective μ sequence was consistently lower than with the equivalent fragment (CμSV*gpt*2) with the μ^a sequence. This was found to be the case in pools of transformants first selected for the *gpt* marker, and also in transfections where the cells were unselected and plated directly into soft agar for the haemolytic assay (see Table 1), which is probably a more accurate determination since frequencies are not distorted by any growth differences during culture. It is possible that the difference in targeting efficiency represents a real effect due to the single nucleotide mismatch with the target, or perhaps other nucleotide substitutions inadvertantly incorporated during the construction of the vector which have not been detected. Waldman and Liskay (29) have studied the effect of nucleotide differences on recombination rates between repeated *tk* genes stably integrated into the genome of mouse L*tk*$^-$ cells and observed a striking 20-fold reduction in efficency with only 2 mismatches in a region of 360 bp. The possibility indicated by our results is that mismatches will also influence the rate of a targeting reaction and this phenomenon deserves further investigation.

The frequency of targeting at the endogenous μ gene in the hybridoma line was in the range of one targeted clone in every one thousand to two thousand transformants. This is substantially less than the frequency of targeted events obtained at the plasmid derived μ gene in the J558*neo*^{R}1S cells with either the CμSV*gpt*2 or CμSV*gpt*6 fragment (1.37% and 0.77% respectively). This difference could be explained on the basis of the above argument, since although the vector DNA is BALB/c derived additional mutations may have accumulated in the μ locus during the culture of the hybridoma. Alternatively this may be a consequence of using two cell lines which have different efficiencies for mediating homologous versus non-homologous recombination. There have been other reports of targeting immunoglobulin sequences in hybridomas (30,31) and these have both involved the use of insertion vectors. In one case (30) the vector was designed to insert into a 2.3 kb region located upstream of the switch region which contained the Ig heavy chain enhancer; targeting was detected by screening for production of a chimeric heavy chain protein containing a human IgG1 constant region, and this occurred at a frequency of one in two hundred transformants. In the other case (31) the vector was designed to target a μ constant region gene containing a two base pair deletion in exon 3, and the frequency detected by complement mediated haemolysis was approximately one in five hundred transformants. Recent evidence suggests that insertion vectors can target more efficiently than replacement vectors (32) which may explain why the frequency we obtain is lower than these values.

Analysis of the targeted loci in the hybridoma cell lines shows that in two cases (clones 6-15 and B2-37) it is likely that vector sequences have concatenated prior to integration. Unexpected integration events using replacement vectors have been studied in detail by Hasty *et al.* (32). In these studies the replacement vectors either recircularized at the position of the double strand break and then integrated as insertion vectors; or joined end to end in a head to tail concatemer and integrated as a replacement vector through the homology at both the ends of the concatemer, or through one end and an internal region of homology. From the data presented here we can exclude the possibility that the replacement vectors integrated as insertion vectors in any of the clones since this event must always leave an uninterrupted copy of the target site, i.e. the 4.3 kb *Xba*I Cμ fragment would still be retained in such targeted clones. End to end joining of repeats of the replacement vector in a head to tail configuration could simply proceed by *in-vivo* religation of the *Eco*RI and *Sal*I cohesive ends of targeting fragments with pUC backbone fragments (the pUC DNA was not purified away after releasing the targeting fragment with *Eco*RI and *Sal*I digestion). However this would be detectable by *Eco*RI digestion since an internal *Eco*RI site would be generated in each repeat of this type of concatemer to give a band corresponding to the unit size of the plasmid standard (13.6 kb). This is not seen, and furthermore a pUC specific probe does not reveal any hybridizing fragments (data not shown) of any size in these clones. Formation of a head to tail concatemer must therefore involve religation of non-compatible ends (*Eco*RI to *Sal*I) or some process of exonucleolytic degradation and then religation; the latter was frequently observed by Hasty *et al.* (32) in an analogous situation. This necessarily involves the loss of some restriction sites at the terminal regions of the targeting fragment and therefore might explain the novel *Xba*I fragment sizes seen in these two clones which are detected with both probes.

The size of the *Xba*I restriction fragment encompassing the Sμ region in the targeted clones indicates the approximate sites of cross-over resolutions in the left arm of the targeting vector (as shown in Figure 3). Since the Sμ region is composed of a highly repeated array of simple sequences (GAGCT, GGGGT), the number of repeats of which would explain the size difference between this region in target and vector DNA, one prediction which we have not observed is the occurrence of slippage in the alignments of target and vector and then crossing over in this area. This would have been apparent by the generation of a

heterogeneous range of sizes of *XbaI* fragments detected with probe A.

Clone F4-2 does not appear to have been generated by a replacement event and nor does the Southern blot data support any other form of vector insertion having occurred at this position. There are two possibilities to account for the allotype conversion: either it was the result of a spontaneous mutation, or there was a recombination event which converted the sequence of the μ locus but the vector DNA then integrated by non-homologous recombination at another position, an event for which there is a precedent (4).

The results described herein demonstrate that this screening approach is sensitive and capable of distinguishing a targeted clone present in a population of transformants at a frequency at least as low as one per cent. The combination of this particular targeting vector and the MB86 monoclonal antibody is only applicable to BALB/c derived hybridomas, but the general principle can probably be extended to other hybridoma cell lines using genes encoding different allotypes in conjunction with different anti-allotype antibodies.

The practical purpose of inserting a *gpt* marker at this position was to facilitate further rounds of targeting at this locus by constructing vectors which would remove the *gpt* marker by homologous recombination and which could therefore be easily selected in 6-thioguanine (since the hybridoma has a *hprt*$^-$ genotype). The aim was therefore to use this strategy with replacement type vectors designed such that they would substitute various regions of the locus including the *gpt* marker by inserts encoding a variety of effector functions for which no obvious genetic selection or screen is normally available. Unfortunately the high rate of loss of the chromosome with the heavy chain locus prevents this being an effective selection for targeted events. The modified targeting vector which contains a cassette with a 5′ deleted *neo* gene will however facilitate a positive genetic selection for additional targeted events using replacement vectors in the second round of targeting which contain a complementary 3′ deleted *neo* gene at one end. In this case *neo*^{R} cells can only be derived following a homologous recombination which will then reconstruct a functional *neo* gene. The effectiveness of this scheme is currently being evaluated.

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